

# Human Dendritic Cells Transfected with RNA Encoding Prostate-Specific Antigen Stimulate Prostate-Specific CTL Responses In Vitro<sup>1</sup>

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Although immunological tolerance to self Ags represents an important mechanism to prevent normal tissue injury, there is growing evidence that tolerance to tumor Ags, which often represent normal peripherally expressed proteins, is not absolute and can be effectively reverted. Prostate-specific Ag (PSA) is a self Ag expressed by both normal and malignant prostatic epithelium, and therefore offers a unique opportunity to examine the ability of self Ags to serve as specific CTL targets. In this study, we investigated the efficacy of autologous dendritic cells (DC) transfected with mRNA encoding PSA to stimulate CTL against PSA Ags in vitro. Ag in form of RNA carries the advantage to encode multiple epitopes for many HLA alleles, thus permitting induction of CTL responses among many cancer patients independent of their HLA repertoire. In this study, we show that PSA mRNA-transfected DC were capable of stimulating primary CTL responses against PSA Ags in vitro. The PSA-specific CTL did not cross-react with kallikrein Ags, a protein, which shares significant homology with PSA, suggesting that harmful autoimmune toxicity may not represent a significant problem with this approach. PSA RNA-transfected DC generated from male or female healthy volunteers or from cancer patients were equally effective in stimulating PSA-specific CTL in vitro, implying that neither natural tolerance to PSA Ags nor tumor-mediated T cell anergy may represent major barriers for CTL generation against the self Ag PSA. This study provides a preclinical rationale for using PSA RNA-transfected DC in active or adoptive immunization protocols. *The Journal of Immunology*, 2000, 164: 5508–5514.

**A** number of prostate-specific and prostate cancer-specific gene products have been identified that contain epitopes, which can be recognized by CTL, thus fostering continued efforts to develop Ag-specific immunotherapy strategies for prostate cancer (1). The most widely and consistently expressed tumor-associated Ag in prostate cancers is prostate-specific Ag (PSA<sup>3</sup>). Because PSA expression is almost exclusively restricted to benign or malignant prostatic epithelium, much research has focused on investigations as to whether or not PSA could represent a relevant rejection Ag recognized by CTL (2–5). The immunogenicity of PSA as a self Ag has been questioned because small amounts of this protein are continuously secreted into the bloodstream, which may lead to tolerance. Furthermore, Ags expressed late during human development and expressed by tumors, which take many years to grow and metastasize, may enhance the likelihood of unresponsiveness to immunotherapeutic intervention. Although uptake and presentation of circulating PSA by professional APC are likely to occur in cancer patients, immunity against PSA is not apparent, suggesting that, in fact, a state of tolerance

exists, potentially imposing major limitations to stimulate therapeutic immune responses in cancer patients. However, there is growing recognition that tolerance to normal proteins is not absolute and can be effectively reverted using properly selected experimental conditions. For example, human dendritic cells (DC) loaded with HLA-restricted peptides isolated from another self protein, namely carcinoembryonic Ag (6), were capable of inducing potent CTL responses in vitro.

Although promising, peptide-loaded DC are capable of presenting single epitopes in the context with specific HLA molecules, most commonly HLA-A2, significantly limiting the patient population eligible for vaccine therapy. Although the efficacy of PSA-specific peptides pulsed onto DC to induce CTL has not been reported to date, a conceptually superior approach would entail the targeting of multiple class I and class II epitopes present on the PSA molecule, which are thought to further stabilize or maintain effective T cell responses *in vivo* (7, 8).

The primary objective of this study was to develop a broadly applicable DC-based immunization strategy for prostate cancer patients. Specifically, we examined the ability of DC transfected with mRNA encoding PSA to stimulate primary PSA-specific CTL responses in vitro. Ag in form of RNA carries the advantage to encode multiple epitopes for many HLA alleles, thus permitting induction of CTL among many cancer patients irrespective of their HLA repertoire (9). Because defective immune responses have been reported in cancer patients (10), we assessed and compared the efficacy of PSA mRNA-transfected DC to stimulate CTL responses among a large number of healthy male and female volunteers and among prostate cancer patients.

In this study, we show that PSA mRNA-transfected DC generated from a large number of individuals reproducibly led to successful in vitro generation of PSA-specific CTL without prior

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<sup>3</sup> Abbreviations used in this paper: PSA, prostate-specific Ag; DC, dendritic cell; GFP, green fluorescent protein; neg, negative; pos, positive.

knowledge of the individual's HLA haplotype. These PSA-specific CTL recognized and lysed cellular targets expressing PSA, but not kallikrein Ags, suggesting that autoimmunity with pathologic consequences may not be a serious problem with this approach. Furthermore, PSA RNA-transfected DC could serve as effective target cells in CTL assays, thus allowing immunological monitoring of PSA responses in humans, while obviating the need for culturing and labeling ex vivo the tumor cells from each patient. CTL responses of similar magnitude were observed among female and male healthy volunteers and cancer patients, suggesting that natural tolerance to PSA or tumor-mediated T cell energy may not compromise the efficacy of this approach. This study provides the experimental foundation for further clinical investigation of a potentially effective and broadly applicable vaccine strategy for prostate cancer patients.

## Materials and Methods

### Human subjects

All cellular material used in these experiments was obtained from human subjects following informed consent through protocols approved by the Investigational Review Board (IRB) at our institution. PBMC were harvested from 10 healthy volunteers and four prostate cancer patients by peripheral blood leukapheresis. Cancer patients were selected based on more aggressive tumor characteristics: Gleason sum  $\geq 7$  (all patients), extraprostatic tumor extension (stage  $\geq$  pT3, all patients), and positive nodal involvement (one patient).

### DC generation from peripheral blood precursors

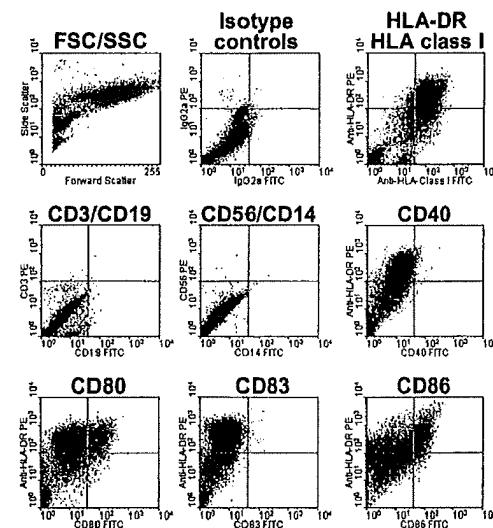
For DC culture, we adopted techniques previously described by Romani (11) or Morse et al. (12), implementing modifications to allow processing of cellular material using defined serum-free media and supplements. Briefly, a concentrated leukocyte fraction was generated through a 2-h restricted peripheral blood leukapheresis processing 6–8 L of blood with each collection. The leukapheresis product was further separated by density-gradient centrifugation over polysucrose/sodium diatrizoate (Histopaque; Sigma Diagnostics, St. Louis, MO), and cells were resuspended in serum-free AIM-V medium (Life Technologies, Grand Island, NY). PBMC were incubated in a humidified incubator for 2 h at 37°C to allow plastic adherence. The adherent cell fraction was used for DC culture by incubation in serum-free AIM-V medium supplemented with human rIL-4 (500 U/ml) and human rGM-CSF (800 U/ml) (R&D Systems, Minneapolis, MN). After 7 days of culture, cells were harvested and phenotypically characterized to assure they met the typical phenotype of immature DC (Fig. 1): CD3<sup>neg</sup>, CD14<sup>neg</sup>, CD16<sup>neg</sup>, CD56<sup>neg</sup>, CD19<sup>neg</sup>, MHC I<sup>pos</sup>, MHC II<sup>pos</sup>, CD40<sup>neg</sup>, CD80<sup>low</sup>, CD86<sup>low</sup>, CD83<sup>neg</sup> (13). DC preparations fulfilling these phenotypic criteria were used for subsequent RNA transfection or pulsing with antigenic peptides or proteins.

### Proteins and peptides

PSA and kallikrein (Sigma) were used in the form of proteins for DC pulsing. Furthermore, the following HLA-A2-restricted peptides were used: influenza virus peptide M1 (FluM1; aa 58–66, GILGFVFTL; Research Genetics, Birmingham, AL) and the PSA-derived peptides PSA-1 (aa 141–150, FLTPKKLQCV) and PSA-3 (aa 154–163, VISNDVCAQV) (kindly provided by J. Schlom, National Cancer Institute, Bethesda, MD). Peptides were dissolved in serum-free AIM-V medium and stored at –20°C.

### In vitro transcription of PSA RNA

To generate in vitro transcribed PSA RNA, the vector pGEM4/PSA/A64 was constructed. In brief, oligonucleotides containing 64 A-T bp followed by a *Spe*I restriction site were cloned into the *Eco*RI and *Nar*I sites of pGEM4Z (Promega, Madison, WI) to create the plasmid pGEM4Z/A64. A 1347-bp *Hind*III fragment containing the entire coding sequence for PSA was isolated from pcDNA3.1 (kindly provided by J. Schlom) and was ligated into the *Hind*III site of pGEM4Z/A64 to generate the plasmid pGEM4Z/PSA/A64. Linearization with *Spe*I followed by in vitro transcription with T7 RNA polymerase (Ambion mMessage mMachine kit, Austin, TX) yields a transcript that contains 9 nt of vector-derived sequence, 36 nt of PSA 5' untranslated region (UTR), 786 nt corresponding to the coding region of PSA, 520 nt of PSA 3' UTR, 51 nt of polylinker sequence, and 64 A nt and 4 nt from the *Spe*I site. To exclude the possibility of anergy to



**FIGURE 1.** Phenotype of cultured, progenitor-derived DC from a prostate cancer patient. DC were generated from the adherent portion of leukapheresis-derived PBMC and cultured for 7 days in serum-free media supplemented with IL-4 and GM-CSF. DC show the typical phenotype of immature DC: CD3<sup>neg</sup>, CD14<sup>neg</sup>, CD16<sup>neg</sup>, CD56<sup>neg</sup>, CD19<sup>neg</sup>, MHC I<sup>pos</sup>, MHC II<sup>pos</sup>, CD40<sup>neg</sup>, CD80<sup>low</sup>, CD86<sup>low</sup>, CD83<sup>neg</sup>. DC preparations fulfilling these phenotypic criteria were used for RNA transfection. The data for a representative patient are presented.

PSA Ags, an irrelevant control RNA encoding jelly fish green fluorescent protein (GFP) was used (14).

### Pulsing of DC

Pulsing of autologous DC with RNA, proteins, or peptides was performed by simple coincubation with DC. In brief, DC were washed twice in PBS, counted, and spun at 300  $\times$  g for 10 min. Subsequently, DC were resuspended at a concentration of  $1 \times 10^6$  cells/ml in AIM-V medium and coincubated for 45 min with either 1.5  $\mu$ g/ml RNA, 25  $\mu$ g/ml peptide, or 20  $\mu$ g/ml protein in a humidified incubator at 37°C/5% CO<sub>2</sub>. RNA, peptide, or protein-pulsed DC were not only used as stimulators for CTL, but also as cellular targets in cytotoxicity assays. For use as targets, RNA-pulsed DC were washed once after antigenic stimulation and incubated overnight to allow expression and presentation of the Ags.

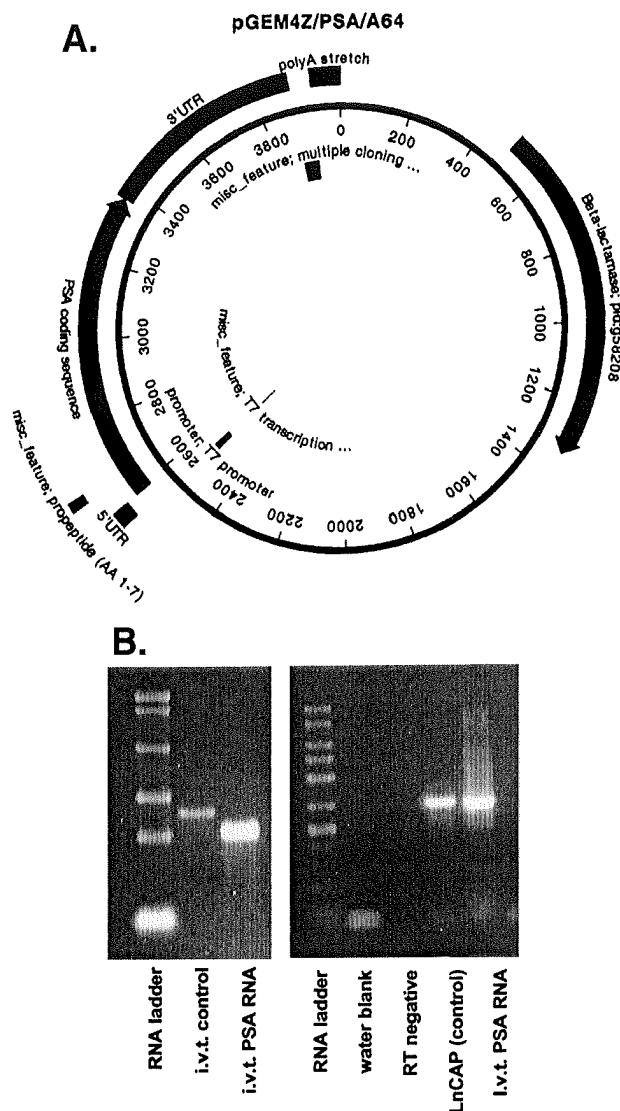
### In vitro cytotoxicity assay

The Ag-presenting function of the pulsed DC was assessed by measuring the induction of primary CTL responses in a standard <sup>51</sup>Cr cytotoxicity assay. The T cell-enriched nonadherent fraction of PBMC obtained following the DC plastic adherence step was used for CTL generation. Non-adherent PBMC were cultured in RPMI cell culture medium supplemented with 20 U/ml human IL-2 and 10 ng/ml human IL-7 (R&D Systems). Cells were stimulated twice, 8 days apart with pulsed autologous DC at a stimulator to effector ratio of 1:10. Following 16 days of culture, effector cells were harvested without further separation and used in microcytotoxicity assays. As determined by flow-cytometric analysis, 77  $\pm$  10% of these effector cells were CD3<sup>pos</sup> and  $\sim$ 40% were of the CD3<sup>pos</sup>/CD8<sup>pos</sup> phenotype. Target cells were labeled with <sup>51</sup>sodium chromate in saline solution (Na[<sup>51</sup>Cr]O<sub>4</sub>; NEN Life Science Products, Boston, MA) by incubation of  $2 \times 10^6$  transfected DC in 1 ml RPMI with 100  $\mu$ Ci of <sup>51</sup>Cr for 1 h at 37°C/5%CO<sub>2</sub>. After three washes,  $5 \times 10^3$  <sup>51</sup>Cr-labeled targets and serial dilutions of effector cells at various E:T ratios were incubated in 200  $\mu$ l of RPMI in 96-well U-bottom plates. These plates were incubated for 6 h at 37°C/5%CO<sub>2</sub>. Then, 50  $\mu$ l of the supernatant was harvested, and released <sup>51</sup>Cr was measured with a scintillation counter. Spontaneous release was less than 15% of the total release by detergent in all assays. SD of the means of triplicate wells was less than 5%.

## Results

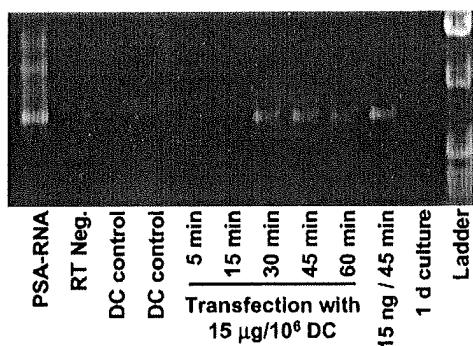
### DC generation and RNA transfection

The goal of these experiments was to develop a sequential protocol for DC generation and RNA loading to produce large amounts of



**FIGURE 2.** In vitro transcription of PSA RNA. *A*, The complete coding sequence of human PSA (kindly provided by J. Schlom) was cloned into the plasmid pGEM4Z/PSA64A, as described in *Materials and Methods*. *B*, PSA RNA produced by amplification of pGEM4Z/PSA/A64 in bacteria and subsequent in vitro transcription (i.v.t.) was subjected to gel electrophoresis and stained with ethidium bromide (*left panel*). To confirm the presence of PSA RNA, a RT-PCR was performed using PSA-specific primers (23; *right panel*). A specific 710-kb band was detected on i.v.t. PSA RNA as well as on the PSA-expressing cell line LNCaP.

PSA RNA-transfected DC from cancer patients. We have adapted the techniques described by Romani (11) and Morse (12), implementing minor modifications to allow human cell processing on a large scale. To develop a clinically compatible protocol, DC were generated from leukapheresis-derived PBMC and cultured for 7 days in low protein, serum-free medium (AIM-V) solely supplemented with GMP-grade GM-CSF and IL-4. The phenotypic characteristics of these DC were similar to those previously described (13), exhibiting the typical phenotype of immature DC: CD3<sup>neg</sup>, CD14<sup>neg</sup>, CD16<sup>neg</sup>, CD56<sup>neg</sup>, CD19<sup>neg</sup>, MHC I<sup>pos</sup>, MHC II<sup>pos</sup>, CD40<sup>neg</sup>, CD80<sup>low</sup>, CD86<sup>low</sup>, CD83<sup>neg</sup> (Fig. 1). The typical yield of PBMC following a 2-h restricted leukapheresis in cancer patients ( $n = 4$ ) and healthy volunteers ( $n = 10$ ) was at least  $3 \times 10^9$  cells, resulting in an average yield of  $3.1 \times 10^8$  viable DC (cancer patients) and  $3.3 \times 10^8$  DC (healthy volunteers) following the

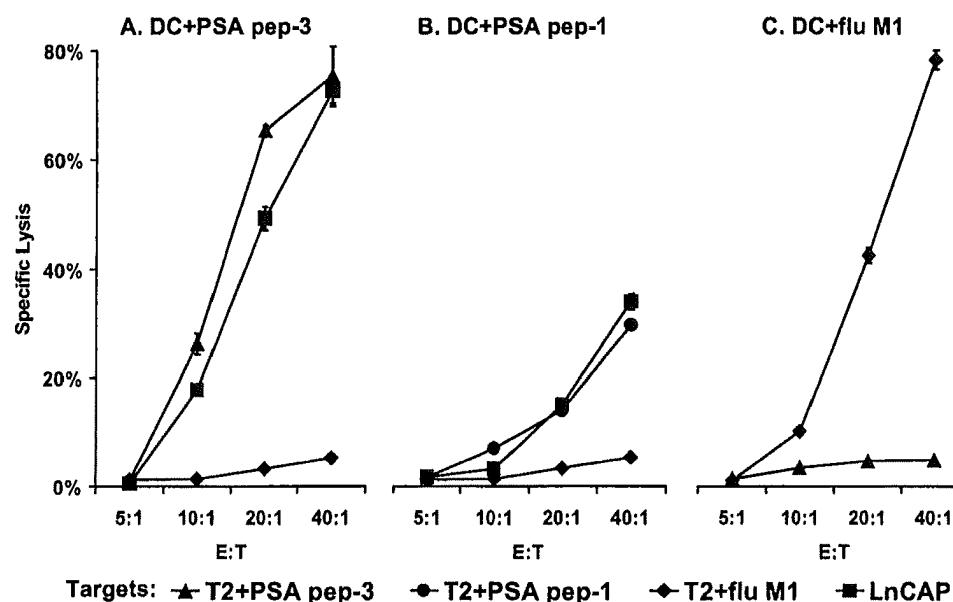


**FIGURE 3.** RNA transfection of DC. DC were transfected with PSA RNA in serum-free medium by coincubation of DC in RNA solution without the use of transfection agents. To demonstrate successful integration, a RT-PCR with PSA-specific primers was performed on RNA prepared from transfected (*lanes 5–11*) and untransfected (*lanes 3 and 4*) DC. The 710-bp PCR product was visualized by gel electrophoresis and ethidium bromide staining following a minimum of 30-min incubation of DC in PSA RNA solution.

7-day culture period. The differences among groups were statistically not significant. We next determined whether these phenotypically immature DC can be successfully transfected with mRNA encoding PSA. PSA mRNA was generated by in vitro transcription of a cDNA plasmid (Fig. 2). Transfection was performed by coincubation of DC with naked RNA without the use of transfection agents used for eukaryotic cell transfection. Incubation of  $1 \times 10^6$  DC with  $1.5 \mu\text{g}$  of PSA RNA for at least 30 min resulted in successful integration and translation of RNA, as evidenced by the successful amplification of the PSA gene product from transfected DC (Fig. 3). Interestingly, even a small amount of  $15 \text{ ng}$  PSA mRNA transfected onto  $1 \times 10^6$  DC for 45 min resulted in detectable PSA expression (Fig. 3). Amplification from transfected DC rather than from adsorbed PSA RNA was demonstrated by the absence of the 710-bp product in DC incubated with PSA RNA for less than 30 min (Fig. 3) as well as by the demonstrated capability of PSA RNA-transfected DC to prime CTL, as presented below. The transient expression of the PSA gene product was demonstrated by complete disappearance of the 710-bp PCR product 24 h following transfection presumably related to intracellular mRNA enzymatic degradation (Fig. 3, *lane 11*). In our hands, RNA transfection of human DC using cationic liposomes was associated with significant toxicity and considerable cell loss (data not shown).

#### DC pulsed with PSA-specific peptides elicit PSA-specific CTL in vitro

To assess the APC function of the DC generated under these conditions, we next determined whether PSA peptide-pulsed DC are capable of stimulating PSA-specific CTL responses in vitro. For these experiments, DC from HLA-A2<sup>+</sup> healthy volunteers were pulsed with the PSA-specific peptides PSA-1 or PSA-3 and used to stimulate CTL from PBMC in vitro. As target cells, peptide-pulsed T2 cells (HLA-A2<sup>+</sup>) or the human prostate cancer cell line LNCaP, which expresses both PSA and HLA-A2, was used. To control for possible anergy to PSA as a self Ag, we tested the ability of DC to stimulate CTL against a HLA-A2-restricted peptide derived from influenza M1 nucleoprotein (flu M1). As shown in Fig. 4, PSA-3 peptide-pulsed DC stimulated a robust CTL response against PSA-3 peptide-pulsed T2 cells and also against human LNCaP cells, while no lysis of M1 peptide-pulsed T2 cells was observed (Fig. 4A). Consistent with studies reported by Correale et al. (2), DC pulsed with PSA-1 peptide were also able, albeit less effectively than PSA-3 peptide-pulsed DC, to induce PSA-specific



**FIGURE 4.** DC pulsed with PSA-specific peptides elicit PSA-specific CTL in vitro. PBMC from a healthy HLA-A2<sup>+</sup>, influenza-vaccinated individual were stimulated with autologous DC pulsed with PSA-3 (A), PSA-1 (B), or flu M1 peptide (C). Induction of primary or flu M1-specific (memory response) CTL was measured in a standard  $^{51}\text{Cr}$  release assay after two stimulations using T2 cells pulsed with PSA-3, PSA-1, flu M1, or LnCaP (HLA-A2<sup>+</sup>, PSA<sup>+</sup>) cells as targets.

CTL responses (Fig. 4B). Stimulation of PBMC with flu M1 peptide-pulsed DC reproducibly generated CTL that lysed flu M1-pulsed, but not PSA-3 peptide-pulsed T2 cells. These data demonstrate that the DC generated under clinical conditions and pulsed with PSA-specific peptides are functionally active by stimulating potent PSA-specific CTL responses in vitro.

*DC transfected with PSA RNA can elicit PSA-specific CTL in vitro*

To test whether human DC can be effectively sensitized with RNA-encoded Ags, we assessed the capability of DC transfected with mRNA encoding PSA to stimulate primary CTL responses in vitro. PSA mRNA was generated by in vitro transcription of the cDNA plasmid pGEM4Z/PSA/A64. DC from a HLA-A2<sup>+</sup> individual were transfected with PSA RNA and used to stimulate CTL responses in vitro against the following targets: 1) human LNCaP prostate carcinoma cells; 2) PSA RNA-transfected DC; 3) PSA-3 peptide-pulsed DC. DC pulsed with the Ags influenza nucleoprotein M1 peptide, or transfected with GFP mRNA were used as control targets. As shown in Fig. 5A, PSA RNA-transfected DC stimulated a robust CTL response, which recognized LNCaP, PSA RNA, or PSA-3 peptide-pulsed DC target cells. DC transfected with the control RNA (GFP RNA) or pulsed with flu M1 peptide were not lysed. The primary nature of this response was suggested by the fact that PSA RNA-transfected DC failed to stimulate significant CTL activity following only one round of stimulation (data not shown). To our knowledge, these findings represent the first demonstration that CTL priming against PSA Ags can be achieved by stimulating PBMC with either PSA peptide-pulsed or PSA RNA-transfected DC. Importantly, these data also demonstrate that PSA RNA-transfected DC can serve as effective targets in CTL assays, thus allowing immunological monitoring of PSA responses in humans, while obviating the need for culturing and labeling the patient's tumor cells (15).

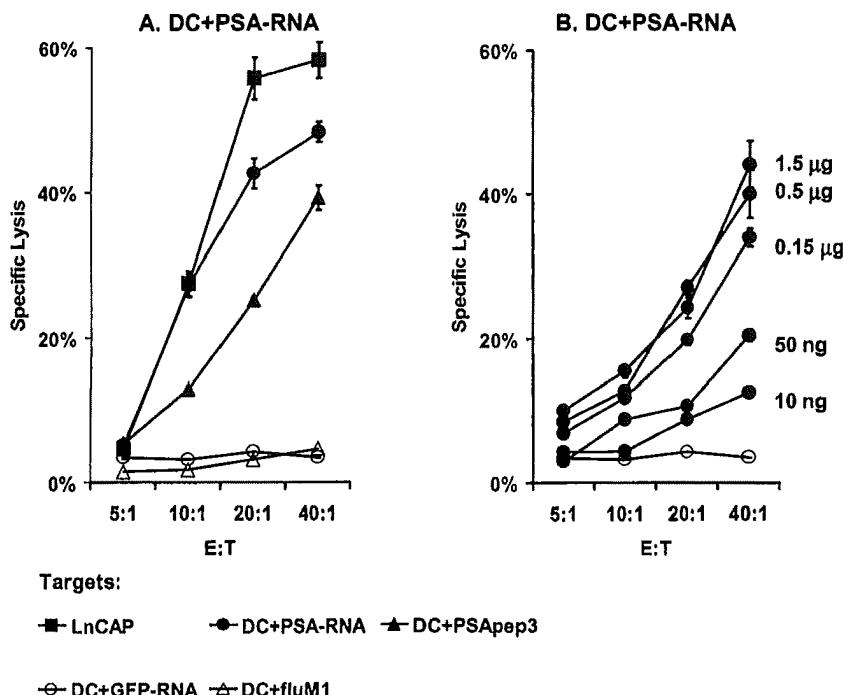
To optimize this immunization protocol and to identify the optimal conditions for RNA transfection, we used increasing amounts of PSA mRNA for DC transfection, followed by the assessment of their ability to stimulate PSA-specific CTL in vitro. PSA RNA and GFP RNA-transfected DC were used as specific or control targets, respectively. As shown in Fig. 5B, a RNA concen-

tration-dependent CTL response was observed. Based on the observed lytic levels, this response was optimal by transfecting  $1 \times 10^6$  DC with 1.5  $\mu\text{g}$  of PSA mRNA (Fig. 5B). Higher RNA concentrations reproducibly did not augment this response (data not shown). In a separate set of experiments, we determined that the function of DC to serve as targets is similarly dependent on the RNA concentration pulsed onto DC. Similar to the previous experiment, DC generated under optimized RNA transfection conditions stimulated CTL, which optimally recognized and lysed DC targets transfected with 1.5  $\mu\text{g}$  of PSA mRNA/ $1 \times 10^6$  DC (data not shown).

*PSA RNA-transfected DC stimulate CTL specific for PSA, but not for the structurally related kallikrein*

Although the use of a well-defined tumor Ag such as PSA may greatly reduce the risk of inducing immune responses against other self Ags, the potential induction of autoimmunity with pathologic consequences remains a concern. In particular, serum kallikrein is a ubiquitous protein found in various normal tissues, which shares about 80% structural homology with PSA (3). The purpose of the following experiment was to investigate whether DC transfected with PSA mRNA activate CTL against epitopes shared with kallikrein. For CTL generation, the following DC preparations were generated: 1) DC transfected with PSA RNA, 2) DC pulsed with PSA protein, or 3) DC pulsed with kallikrein protein. These DC were used both as stimulators and as targets in cytotoxicity assays. Similar to the previous experiments shown in Fig. 5, PSA RNA-transfected DC (Fig. 6A) and also PSA protein-pulsed DC (Fig. 6B) were capable of stimulating CTL responses recognizing and lysing DC targets presenting PSA, but not kallikrein Ags, suggesting the absence of CTL recognizing epitopes shared between these structurally related proteins. Interestingly, as shown in Fig. 6C, kallikrein protein-pulsed DC were also capable of stimulating kallikrein-specific, but not PSA-specific CTL, despite the fact that kallikrein is a ubiquitously expressed self Ag. These experiments show that DC transfected with PSA RNA can stimulate CTL responses, which are specific for PSA Ags and do not cross-react with kallikrein, suggesting that the induction of harmful autoimmunity may not be a serious issue with this approach.

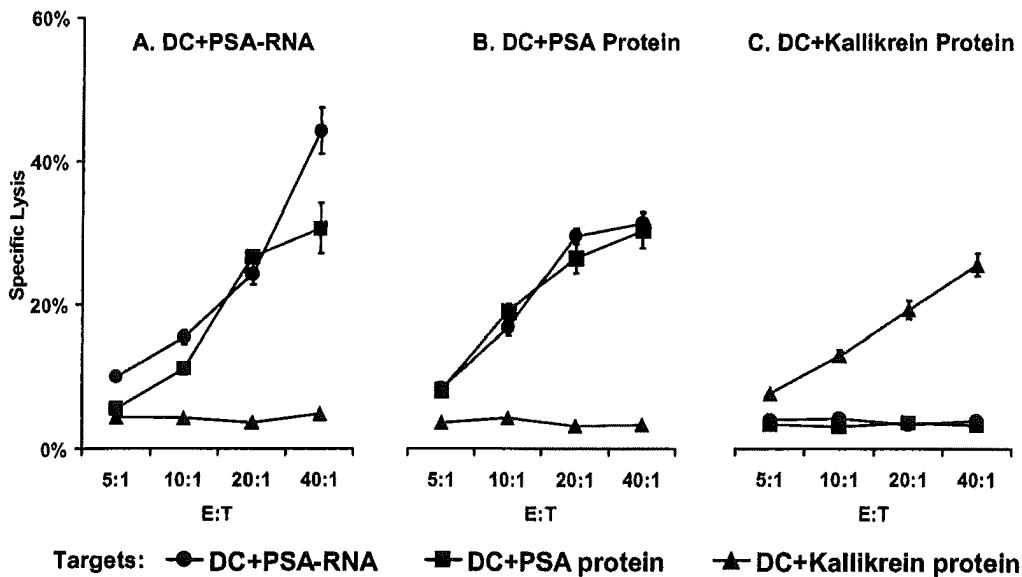
**FIGURE 5.** DC transfected with PSA RNA can elicit PSA-specific CTL in vitro. *A*, Autologous PBMC from an HLA-A2<sup>+</sup> male were stimulated twice with autologous DC transfected with PSA RNA. PBMC were then assayed for the presence of PSA-specific CTL recognizing and lysing HLA-A2<sup>+</sup>, PSA<sup>+</sup> human LNCaP cells, DC transfected with PSA-3 peptide, DC transfected with GFP RNA, and DC pulsed with flu M1 peptide. *B*, Autologous DC ( $2 \times 10^6$ ) were transfected with increasing amounts of PSA RNA, as shown above. RNA-transfected DC were used to stimulate CTL from PBMC. These CTL were then assayed for lytic activity against DC transfected with PSA RNA.



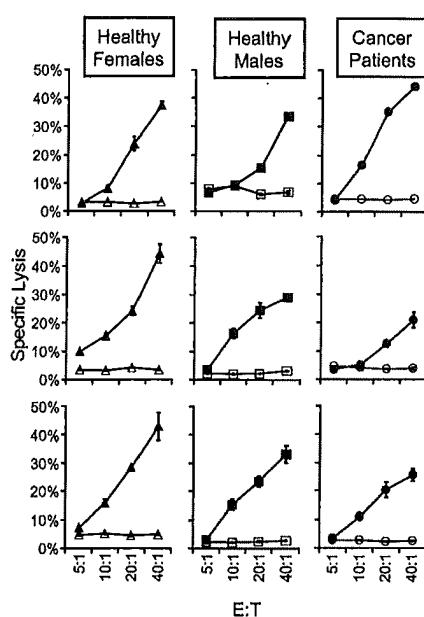
*Stimulation of PSA-specific CTL by PSA RNA-transfected DC: no evidence for immunological tolerance or tumor suppression*

The experiments described to date have demonstrated that DC transfected with PSA RNA are capable of inducing CTL responses in HLA-A2<sup>+</sup> healthy volunteers. The concern was raised that tumors could tolerize the immune system against Ags expressed on the tumor cells (16). Therefore, cancer patients may not respond to a particular vaccine strategy in a similar fashion as healthy volunteers. The purpose of the following experiments was several-fold. First, we sought to assess the efficacy of PSA RNA-transfected DC to stimulate primary CTL responses among cancer patients. Sec-

ond, to assess the general applicability of this vaccine strategy, we tested whether in vitro stimulation of PSA-specific CTL can be achieved from a large number of randomly selected individuals irrespective of their HLA composition. Finally, we tested whether males exhibit immunological tolerance to PSA by comparing the ability to stimulate CTL between males and females. DC were generated from a total of 14 randomly selected individuals consisting of 4 healthy males, 6 healthy females, and 4 prostate cancer patients. Following PSA mRNA transfection, DC from all groups were analyzed and compared for their ability to generate PSA-specific CTL responses. Fig. 7 demonstrates the results from three



**FIGURE 6.** DC transfected with PSA RNA elicit CTL specific for PSA, but not for kallikrein Ags. Autologous DC were loaded with PSA RNA (*A*), PSA protein (*B*), and kallikrein protein (*C*), and used as stimulators to generate CTL. Cytotoxicity was assayed in a standard  $^{51}\text{Cr}$  release assay using PSA RNA-transfected DC, PSA protein, and kallikrein protein-pulsed DC as targets.



**FIGURE 7.** DC transfected with PSA RNA can stimulate PSA-specific CTL in prostate cancer patients. The ability of PSA RNA-transfected DC to stimulate CTL among randomly selected healthy volunteers and cancer patients was analyzed. DC were generated from 14 individuals consisting of 6 healthy females, 4 healthy males, and 4 prostate cancer patients. Following transfection with PSA RNA, DC were used to stimulate PSA-specific CTL responses in vitro, as measured in standard  $^{51}\text{Cr}$  release assays. As targets, PSA RNA-transfected (filled symbols) and GFP RNA-transfected (open symbols) DC were used. The figure shows the results from three representative candidates within each group.

representative candidates within each group. Although minor variances in the lytic levels were observed among and within the three groups, we could reproducibly generate CTL responses against PSA Ags from all individuals tested. Interestingly, CTL responses of a similar magnitude were observed between healthy males and cancer patients. Thus, we find no evidence that the T cell population of healthy males or cancer patients is deficient in PSA-specific CTL precursors.

## Discussion

In this study, we demonstrate that autologous DC transfected with RNA encoding PSA are capable of stimulating CTL responses in vitro in a large number of randomly selected individuals without prior determination of their HLA phenotype. The advantages of stimulating CTL by using RNA-loaded DC are 3-fold: First, unlike synthetic peptides encoding HLA-restricted epitopes, RNA spanning the entire coding sequence for PSA may encode multiple epitopes corresponding to many HLA alleles. Second, we have demonstrated that RNA-transfected DC stimulate CTL that recognize and lyse human PSA-expressing LNCaP cells with similar efficacy as PSA RNA-transfected DC. This suggests that RNA-transfected DC can function as specific targets in CTL assays, providing a general platform to monitor CTL responses by obviating the need of culturing and labeling tumor cells as targets (14). Third, as demonstrated in this study, RNA-pulsed DC vaccines can be used to stimulate CTL from virtually all cancer patients regardless of their HLA composition. PSA is a tissue-specific gene product, which shares strong homology with several regions of normal tissue kallikrein proteins. Hence, stimulation of CTL responses against PSA can induce potentially harmful autoimmune toxicity.

However, as shown in this manuscript, the CTL stimulated by PSA RNA-transfected DC are strictly PSA specific and do not cross-react with kallikrein epitopes. Although this study implies that the PSA-specific CTL do not cross-react with normal proteins such as kallikrein, the question as to whether or not autoimmunity may represent a serious problem with this approach remains to be carefully evaluated in a clinical trial setting.

It was argued that tumors could tolerize the immune system against Ags expressed on tumor cells (17). In one study, tumor Ag-specific CD4<sup>pos</sup> T cell anergy was observed in tumor-bearing mice as an early event in the course of tumor progression (16). In contrast, others have not found any impairment of tumor Ag-specific CD8<sup>pos</sup> responses (18, 19). In this study, we have not seen evidence that in vitro stimulation of CTL against the self Ag PSA is significantly blunted in cancer patients as compared with healthy volunteers. Although this could be attributed to an insufficient level of tumor burden in the patients examined, the issue as to how or to what extent progressing tumors impact negatively on tumor-specific immunity is yet unresolved and awaits further investigation.

Ags presented by thymic APC cause clonal deletion of autoreactive T lymphocytes, whereas the response to peripherally expressed proteins with no access to the thymus can result in either their physical elimination or functional anergy. Because healthy women lack significant PSA expression, whereas healthy males or prostate cancer patients are exposed to higher levels of circulating PSA, we initially hypothesized that significant differences may exist in our ability to induce CTL against PSA among these groups. Surprisingly, we found that PSA RNA-transfected DC generated from healthy males, females, and cancer patients stimulated CTL responses of similar magnitude. One possible explanation is the fact that PSA expression, albeit at very low levels, can be found in some tissues in normal females (20, 21). Indeed, anti-PSA Abs have been detected in some healthy females, suggesting that PSA can be the target of a humoral autoimmune response (22). The present study shows that PSA-specific CTL can be generated to a comparable extent from healthy males and females, or from prostate cancer patients, suggesting that PSA-specific CTL precursors are present at comparable frequency among those groups, which can be activated by stimulation with PSA RNA-transfected DC. Although in this study we could not identify any evidence for immunological tolerance or tumor-mediated anergy to PSA, the critical questions remain as to whether the increase in the observed PSA CTL frequency will ultimately lead to a clinical benefit for cancer patients. Such questions will need to be carefully determined in a clinical trial setting.

## Acknowledgments

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